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Cloning and characterization of the *Drosophila* presenilin homologue

Gabrielle L. Boulianne,[1,CA] Izhar Livne-Bar,[1,2] James Michael Humphreys,[1] Yan Liang,[2] Cong Lin,[2] Evgeny Rogaev[2] and Peter St. George-Hyslop[2]

[1]Division of Neurology and Program in Developmental Biology, Hospital for Sick Children, Department of Physiology and Zoology, University of Toronto, 555 University Avenue, Toronto, Ontario M5G 1X8; [2]Center for Research in Neurodegenerative Disease, Department of Medicine (Neurology), The Toronto Hospital and Department of Medicine, University of Toronto, 6 Queen's Park Crescent, Toronto, Ontario M5S 3H2, Canada

[CA]Corresponding Author

MUTATIONS in two genes, *PS1* and *PS2*, coding for the presenilins, have been linked to the early onset form of familial Alzheimer's disease (AD). Here we report the identification of a *Drosophila melanogaster* homologue of human *PS* genes, *Dps*, which maps to band 77B-C on chromosome 3 and is expressed at multiple developmental stages. The predicted amino acid sequence of the *Dps* product is 53% identical to human presenilins, with the greatest similarity in the putative transmembrane domains, the hydrophobic domains at the beginning and the end of the cytoplasmic TM6-TM7 loop and the C-terminus. Analysis of *Dps* in a genetically tractable model system such as Drosophila may provide insight into the mechanisms of Alzheimer's disease (AD) necessary for the development of rational therapeutic approaches.

Key words: Alzheimer's disease; *Drosophila*; Presenilin

Introduction

Alzheimer's disease (AD) is a degenerative disorder of the CNS which causes progressive memory and cognitive decline during mid to late adult life. The disease is characterized by various neuropathological features including extracellular amyloid plaques and intraneuronal neurofibrillary tangles. An approach which has been successfully used to identify causative factors in AD has come from studying cases which are inherited as in autosomal dominant trait. This approach has led to the identification of three human genes, coding for amyloid precursor protein (*APP*)[1] and presenilins 1 and 2 (*PS1* and *PS2*)[2–4] for which missense mutations have been associated with early onset familial AD. Mutations in the *βAPP* gene account for < 3% of families with disease onset before 65 years of age.[1] In contrast, mutations within the *PS* genes are more common. The first human presenilin gene cloned, *PS1* maps to chromosome 14.[2] Missense mutations in this gene are associated with the most aggressive subtype of AD and account for up to 50% of early onset familial AD cases.[5–7] The second gene, *PS2*, maps to chromosome 1 and is associated with a form of familial AD with onset between 50 and 70 years of age and a slower rate of progression.[3,4] Analysis of the cDNA sequences of both genes predicts proteins with at least seven putative transmembrane (TM) domains, and large hydrophillic cytoplasmic domains at the N-terminus and between TM6 and TM7.[8]

At present, the function of the vertebrate presenilin genes is unclear. Immunocytochemical results have demonstrated that the presenilin proteins are located within intracellular organelles, primarily in the endoplasmic reticulum,[9,10] suggesting a role in protein processing/trafficking. Consistent with this model, mutations in presenilins have recently been found to alter the processing of βAPP in both plasma and fibroblasts derived from patients with AD,[11,12] in transfected cells[13] and in transgenic mice,[13,14] resulting in an increase in extracellular levels of β-amyloid.

Homologues of presenilins have also been identified in *Caenorhabditis elegans*.[15,16] *spe-4*, which is 26% identical to human presenilins, is expressed in a Golgi-derived membrane-bound organelle in *C. elegans* spermatocytes, where it is thought to be involved in membrane/protein trafficking.[16] *sel-12*

(50% identical) was identified through a genetic screen for mutations in genes capable of suppressing the phenotype caused by expression of an activated *lin-12* gene. *lin-12* is the *C. elegans* homologue of the *Drosophila* gene *Notch*, which encodes a highly conserved TM protein thought to specify the developmental fate of many cells in most complex organisms.[17] Whether *sel-12* is involved directly in *Notch/lin-12* signalling or in some aspect of its processing remains to be determined but any link between presenilin and the *Notch/lin-12* signalling pathway would provide valuable insight into the function of presenilin.

Here, we report the cloning and characterization of a *Drosophila* homologue of human presenilins, Dps. This protein shares 53% sequence identity with human presenilins, 48% with the *sel-12* product and 18% with the *spe-4* product. Low stringency southern hybridization techniques suggest the presence of a single gene in *Drosophila* which maps to chromosome 3, between bands 77B-C. *Dps* produces multiple transcripts whose expression are spatially and temporally controlled during development. The characterization of a *Drosophila* homologue of presenilin provides us with a valuable tool to study its function and relationship to the *Notch* pathway, and the biological differences between *C. elegans* and *Drosophila* might provide a comprehensive view of gene function. Furthermore, the availability of powerful genetic approaches in *Drosophila* should permit the development of an animal model to examine the role of presenilins in Alzheimer's disease.

Materials and Methods

Complementary DNA cloning and Southern analysis: Redundant oligonucleotides (1105: 5'-CTNCCN-GARTGGACNGYCTGG and 1106: 5'-RCANGC-(AGT)ATNGTNGTRTTCCA) were designed from published nucleotide sequence data[22,3,15] for highly conserved regions of the presenilin/sel-12 proteins ending/beginning with Trp (e.g. at residues Trp247 and Trp404 in presenilin 1 and at Trp253 and Trp385 in presenilin 2). These primers were used for reverse transcriptase polymerase chain reaction (RT-PCR) of mRNA from adult and embryonic *D. melanogaster*. The products were then reamplified using internal conserved redundant primers 1107: 5'-TTTTTT-CTCGAGACNGCNCARGARAGAAAYGA and 1108: 5' TTTTTTGGATCCTARAA(AGT)ATRA-ARTCNCC. A 600 bp product was then subcloned into pBS, sequenced, and shown to contain an open reading frame (ORF) with a putative amino acid sequence highly homologous to that of the human presenilins. This fragment was then used to screen 500 000 plaques of a cDNA library in ~gt10, made by L. Kauvar (E6 and E7, 3–12 h embryonic) and six independent clones of approximately 2 kb were isolated. The nucleotide sequences of these clones were determined, and the putative amino acid sequence predicted by the longest ORF was deduced and compared with that of the human presenilins.

To determine whether additional genes coding for presenilin homologues existed in *Drosophila* we probed genomic DNA with a full length *Dps* cDNA under low and high stringency (low = 1 M NaCl, 0.5% SDS for 1 h at 55°C; high = 0.2 × SSC, 0.5% SDS for 1 h at 65°C). We found no evidence for cross-hybridizing bands under conditions which would detect > 55% DNA sequence identity.

Fly stocks and chromosomal in situ hybridizations: Fly cultures were maintained according to standard procedures at 25°C. Embryos and larval tissues were obtained from Oregon-R flies. The deficiency Df(3L)ri-79c/TM3 is described in Lindsley and Zimm[18] and was obtained from the Bloomington Stock Center. The chromosomal location of the *Dps* gene was mapped by *in situ* hybridization to squashed polytene salivary gland chromosomes using a full length biotinylated cDNA probe (Detek, Enzo Biochemicals) essentially as described.[19]

Northern analysis: A total of 2.5 µg poly(A)+ mRNA from 18 h embryos, 4-day-old larvae and adult *Drosophila* (Clonetech) was electrophoresed, blotted and probed with a full length *Dps* cDNA as described previously.[20]

In situ hybridization to RNA in whole-mount embryos or third instar larval imaginal discs: *In situ* hybridization to whole mount embryos and larval imaginal discs was carried out following the method of Hughes *et al*,[21] using fluorescein-labelled RNA (Boehringer-Mannheim) synthesized from the full-length Dps cDNA. No hybridization was detected using control (sense) probes or when DNA probes were omitted (data not shown).

Results and Discussion

Comparison of the predicted amino acid sequences for the human PS1 and PS2 proteins with that predicted for the *C. elegans* sel-12 protein revealed that the highest degree of amino acid sequence conservation corresponded to residues 213–291 and 375–435 of human PS1 (87% and 84% amino acid sequence identity between PS1, PS2 and sel-12).[2,3,15] These residues were only weakly conserved however in *C. elegans* spe4 (less than 28% sequence identity).[16] To isolate a close homologue of the human presenilins and the

C. elegans sel-12 transcripts, nested redundant oligonucleotide primers corresponding to these sequences were used to recover a single RT–PCR product from RNA isolated from *D. melanogaster* 6–12 h embryos or adults. This RT–PCR product, which contained an ORF with substantial homology (>90%) to human PS1 at the initial 20 and the terminal 15 amino acid residues, was then used to screen an embryonic *D. melanogaster* cDNA library under conditions of moderate stringency. Multiple clones were identified: two were identical and contained a larger 5′ UTR than the four remaining clones. The longest ORF was detected in clone dps13-1 and predicts a sequence of 541 amino acids from the first in-phase ATG codon (accession number: U77934). The four remaining clones are predicted to have identical ORFs containing 527 amino acids, and differ from the longest clones by the absence of 42 nucleotides (1239–1280 bp) predicted to encode an additional 14 amino acids (residues 397–384).

To confirm that *Drosophila* has a single presenilin gene we performed Southern blotting experiments in which we probed genomic *Drosophila* DNA with a full length *Dps* cDNA under low and high stringency. We found no evidence for cross-hybridizing bands under conditions which would detect >55% DNA sequence identity. We hypothesize that anything less than 55% identical would be unlikely to have redundant functions and therefore would not interfere with our analysis of *Dps*. The chromosomal location of the *Dps* gene was then mapped to a locus on the left arm of the third chromosome at band 77B–C (data not shown). This localization was further confirmed by hybridization of the *Dps* cDNA to genomic P1 clones (#DS03069 obtained from the Berkeley *Drosophila* genome project) which encompasses the chromosomal bands 77A3–77B3 and by Southern analysis which demonstrated that the *Dps* gene is included within the deficiency, Df(3L)ri-79c/TM3, with breakpoints at 77B–C;77F–78A.

Comparison of the predicted amino acid sequence of the longest ORF with that of human PS1 revealed 53% overall sequence identity. Residues predicted to compose TM domains or membrane-associated domains (especially TM1, TM3, TM5, TM6, TM7, the hydrophobic domains at the beginning and the end of the TM6–TM7 loop domain, and the C-terminus) showed a much higher level of sequence identity (Fig. 1). Interestingly, 16 of 20 residues mutated in human PS1 or PS2 and giving rise to human familial AD are conserved in Dps. In addition, the cysteine residue which is mutated in the *C. elegans sel-12* gene is also conserved. This suggests that these residues form an important functional or structural domain of the presenilins.

There is no sequence conservation at the N-terminus or at the apex of the TM6–TM7 loop domain, consistent with the observation that there is a lack of homology in those domains between PS1 and PS2, and between the presenilins and sel-12. The 42 nucleotide insertion observed in clone dps13-1 is predicted to result in the addition of 14 amino acids at the apex of the TM6–TM7 loop domain. We have yet to determine whether this sequence variation is because of alternative splicing. The boundaries of the 14 amino acid insert do not correspond to known intron–exon boundaries within the human PS1 or PS2 genes however, the intron–exon boundaries encoding residues in the apex of the human TM6–TM7 loop domains are not conserved either.[22,23] Nevertheless,

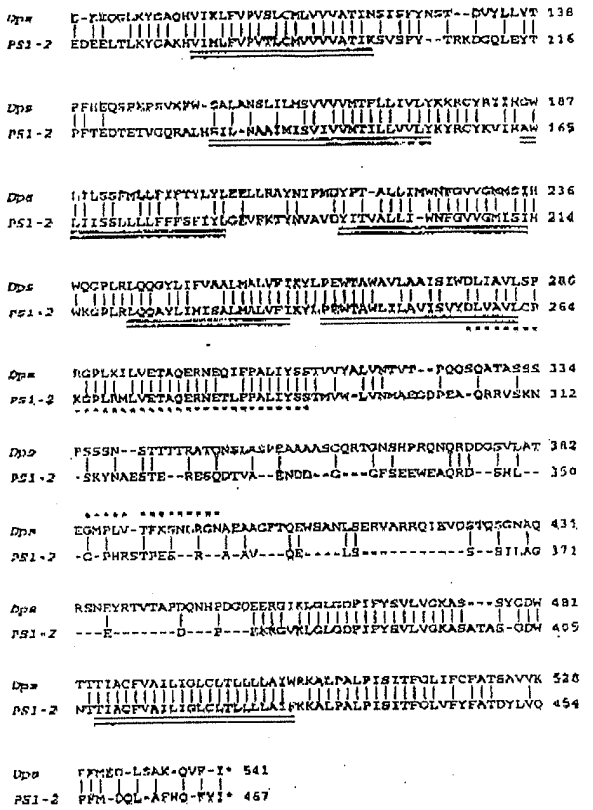

FIG. 1. Comparison of the predicted amino acid sequences for *Dps* with human presenilin 1 (PS1) protein (bottom line) using single-letter amino acid codes. Residue numbers are provided at the end of each line. Amino acid sequence identity is indicated by a vertical line. Putative transmembrane domains (based upon Kyte–Doolittle analysis and experimental observations[9] on the orientation of exposed domains in human PS1) are included within horizontal parallel lines. Residues in human *PS1* exon 4 and human *PS1* exon 9 which are affected by alternative splicing in the human PS1 transcript are depicted by subscripted asterisks. Residues affected by putative alternative splicing of the *Dps* transcript are depicted by superscripted asterisks.

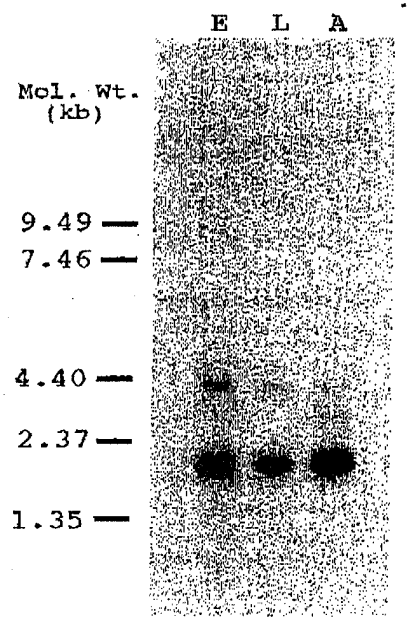

FIG. 2. Blot hybridization of 2.5 μg *Drosophila* poly(A)+ mRNA from embryos (lane 1), larvae (lane 2) and adult (lane 3) was probe with a full length *Dps* cDNA. Positions of mol. wt ([x]10^{-3}) markers are shown at left.

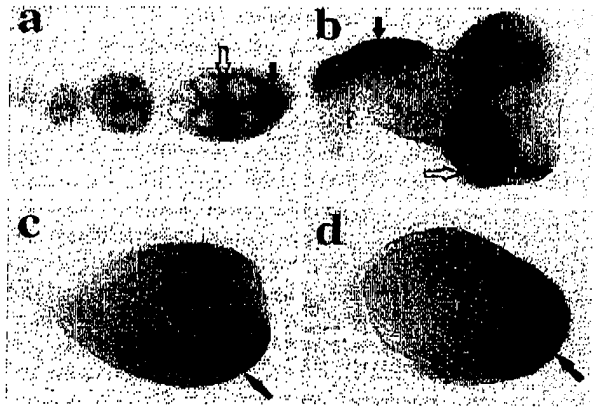

FIG. 3. *In situ* hybridization to Dps in *Drosophila* oocytes and imaginal disc. Antisense probes to *Dps* revealed high levels of mRNA during oogenesis (a) in the nurse cells (outlined arrow) and the developing oocyte (black arrow). (b) In third larval instar imaginal discs, *Dps* transcripts accumulate in the eye-antennae disc at a region that will give rise to the postoccipital sensilla of the antenna (black arrow), and in the eye disc, in progenitors of the ocellus and the ocellar and interocellar bristles (outlined arrow). We also detected strong hybridization signals in the posterior part of the leg discs in areas fated to develop into the adult femur and tibia (c,d).

alternative splicing of transcripts encoding residues in the TM6–TM7 loop has been detected in both human PS1 (residues 257–290) and human PS2 (Glu 325).[22,23] We have previously speculated that the alternative splicing of the human PS1 TM6–TM7 loop domain may have functional significance by altering the size and structure of this loop domain.[3,23] This functional importance is supported by the observation that splice site mutations affecting human PS1 Exon 10, which cause in-frame fusion of Exon 9 to Exon 11 and deletion of residues 290–319 at the apex of the TM6–TM7 loop of human PS1, are associated with early-onset AD. Consequently, if the difference in the sequence of the ORF between clone dps13-1 and the remaining full-length clones does arise from physiological alternate splicing, then this splicing event is likely to have relevance to the biological function of the *Drosophila* presenilin homologue *Dps*.

The temporal pattern of *Dps* expression was examined by Northern blot analysis (Fig. 2). Poly(A)+ mRNA derived from *Drosophila* embryos, larvae and adult was probed with a full-length *Dps* cDNA under stringent conditions. Several transcripts ranging from 2 to 5 kb were detected at all developmental stages, with a major transcript (2 kb) most abundant in adults and a minor transcript (~4 kb) most abundant in embryos, but detected at all stages. In addition we observed stage-specific transcripts including a 5 kb transcript in embryos and a 3.5 kb transcript in adults. These may result from alternative splicing or transcript initiation or termination. In human presenilins, low abundance, high mol. wt transcripts (~7.5 kb) can be detected in many tissues[2,3] and appear to arise from alternative polyadenylation sites.[25]

To determine the spatial pattern of *Dps* expression, we examined whole mounted ovaries, embryos and third instar larval imaginal discs after *in situ* hybridization with RNA antisense probes derived from a full length *Dps* cDNA (Fig. 3). We detected high levels of *Dps* RNA during oogenesis in the nurse cells and the developing oocyte (Fig. 3a). *Dps* transcripts were also uniformly distributed in the blastoderm stage embryo; this probably represents maternal RNAs which have accumulated during oogenesis (data not shown). In third instar larval imaginal discs, which will give rise to adult structures, *Dps* expression was restricted to the eye-antennae and the leg discs. In the eye-antennae disc, *Dps* RNA accumulated in the precursor of the postoccipital sensilla of the antennae and in areas of the eye disc corresponding to progenitors of the ocellus and the ocellar bristles (Fig. 3b). In the leg discs, *Dps* expression was restricted to the posterior compartment, specifically in areas that would give rise to the adult tibia and femur (Fig. 3c,d). This dynamic expression pattern of *Dps* suggests that it may have multiple roles during *Drosophila* development.

Conclusion

We have identified and characterized a *Drosophila* homologue of human presenilins which are a highly conserved, novel family of proteins. Unlike vertebrates, there is a single presenilin gene in *Drosophila* which should facilitate the analysis of its function using molecular and genetic approaches. *Dps* produces several transcripts which are dynamically expressed during development. Whether individual transcripts give rise to different isoforms of Dps with distinct functions or cellular localizations, remains to be determined. The relationship between Dps and the Notch signalling pathway also needs to be investigated in *Drosophila* where the pathway is well characterized. The recent discovery of mutations in human *Notch 3* associated with the adult-onset neurological disease CADASIL[25], suggests that the analysis of presenilin and its relationship to the Notch signalling pathway may provide valuable insights into AD and other adult-onset neurological diseases in humans.

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General Summary
Alzheimer's disease (AD) is a degenerative disorder of the central nervous system which causes progressive memory and cognitive decline during mid to late adult life. Recently, mutations in two genes, *PS1* and *PS2*, have been linked to the early onset form of familial AD. This discovery provides a unique opportunity to generate animal and cellular models with which to investigate the biochemical pathways leading to neuronal death in AD. Here we describe the isolation and characterization of a *Drosophila* homologue of the presenilins. The analysis of presenilin in a simple organism such as *Drosophila* will provide insight into its normal function during development. In addition, the analysis of mutated presenilins may allow us to develop an animal model which may in turn provide insights into the mechanisms of AD necessary for the development of rational therapeutic approaches.